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(54) Title: MUTANT MONO-OXYGENASE CYTOCHROME P450cam

(57) Abstract

A mutant of the mono-oxygenase cytochrome P450cam in which the cysteine residue at position 334 is removed.

MUTANT MONO-OXYGENASE CYTOCHOME P450cam

The present invention relates to a mutant of the mono-oxygenase cytochrome P-450cam.

Mono-oxygenases catalyse the selective oxidation of activated and unactivated carbon-hydrogen bonds using oxygen[1], and are therefore of great interest for potential use in organic synthesis. However, progress in this area has been hampered by the difficulty in isolating sufficient quantities of the mono-oxygenase enzyme and/or the associated electron-transfer proteins. Despite the availability of amino acid sequences of more than 150 different cytochrome P-450 mono-oxygenases, to date structural date of only three are available[2,3,4], and few have been successfully over-expressed in bacterial systems[5].

One cytochrome P-450 mono-oxygenase, which is soluble and can be expressed in sufficient quantities, is the highly specific P-450cam from P. putida which catalyses the regio- and stereo-selective hydroxylation of camphor to 5-exo-hydroxycamphor[6]. The high resolution crystal structure of P-450cam has been determined[2], and since the mechanism of action of this bacterial enzyme is believed to be very similar to that of its mammalian counterparts, it has been used as a framework on which structural models of mammalian enzymes are based.

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The nucleotide sequence and corresponding amino acid sequence of P-450cam have been described[5,7]. The location of an active site of the enzyme is known and structure-function relationships have been investigated[8,9]. Mutants of P-450cam have been described at the 101 and 185 and 247 and 295 positions[9,10,11] and at the 87 position[12]. A mutant in which tyrosine 96 (Y96) has been changed to phenylalanine 96 (the Y96F mutant) has been described[11,13,14,15]. But in all cases the papers report effects of the mutations on the oxidation reactions of molecules which had previously been shown to be substrates for the wild-type enzyme. There is no teaching of how mutations might be used to provide biocatalysts for oxidation of different, novel substrates.

In an attempt to develop new biocatalysts, we have initiated a project which aims to redesign P-450cam, such that it is able more effectively to carry out specific oxidations of organic molecules whether or not these are substrates for the wild-type protein.

The three dimensional structure of P-450cam shows the active site to provide close van der Waals contacts with the hydrophobic groups of camphor as shown in Figure 1. Of particular significance are the contacts between camphor and the side chains of leucine 244, valine 247 and valine 295. Three aromatic residues (Y96, F87 and F98) are grouped together and line the substrate binding pocket, with a hydrogen bond between tyrosine 96 and the camphor carbonyl oxygen maintaining the

substrate in the correct orientation to ensure the regio- and stereo- specificity of the reaction.

Lipscomb and co-workers[16] demonstrated in 1978 that wild-type P-450cam showed a propensity to dimerise, but they also reported that the catalytic activity of the monomer and dimer towards camphor oxidation were indistinguishable. Since the dimerisation reaction could be reversed by thiol reducing agents, they concluded that it occurred by intermolecular cysteine disulphide (S-S) bond formation. They were unable to determine whether dimerisation involved more than one cysteine per P-450cam molecule. Nor were they able to identify the key cysteine residue(s) involved in this reaction because neither the amino acid sequence nor crystal structure of P-450cam were known at the time.

We used molecular modelling to investigate the likely effects of points mutations to the three aromatic residues (Y96, F87, F98) in the active site pocket. We noted that replacement of any of these aromatic residues with a smaller, hydrophobic non-aromatic side-chain could provide an "aromatic pocket" which could be used to bind more hydrophobic substrates. The program GRID[17] was used to calculate an energy of interaction between an aromatic probe and possible mutants of cytochrome P-450cam where these residues were changed to alanine (F87A, Y96A and F98A). The results were then examined graphically using the molecular modelling package Quanta[18].

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The mutant F98A appeared to have the strongest binding interaction within the active site cavity accessible to the aromatic probe, with that of Y96A being slightly smaller, and that of F87A being substantially less. It was decided in the first instance to mutate tyrosine 96 to alanine as it is more central to the binding pocket, whereas phenylalanine 98 is in a groove to one side. Also, removal of tyrosine 96 should decrease the specificity of the enzyme towards camphor due to the loss of hydrogen bonding to the substrate.

According to one aspect of the present invention a mutant of the mono-oxygenase cytochrome p-450cam is provided in which the cysteine residue at position 334 is removed.

Preferably the removal is by the substitution of another amino acid except cysteine for the cysteine residue.

Alternatively the removal is by the deletion of the entire cysteine 344 residue from the enzyme.

Suitably the tyrosine residue at position 96 in the mutant is replaced by the residue of any amino acid except tyrosine.

Conveniently the amino acid is selected from any one of the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine except that in the

case of the cysteine residue at position 334, the amino acid is not cysteine and in the case of the tyrosine residue at position 96 the amino acid is not tyrosine.

Preferably the amino acid residue at one or more of the positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396 is replaced by another amino acid residue.

We examined the structure of P-450cam generated from the published crystallographic atomic co-ordinates using the modelling programme Quanta. We determined that there are five cysteines near the surface of P-450cam (cysteines 58, 85, 136, 148, 334) which might participate in intermolecular disulphide bond formation leading to protein dimerisation. We carried out sit-directed mutagenesis to substitute each of these cysteines to alanine, thus generating five Cys - Ala surface mutants.

The extent of protein dimerisation in the wild-type P-450cam and the five surface Cys - Ala mutants were investigated, The presence of dimer was detected by both anion exchange fast protein liquid chromatography on a Resource Q column (Pharmacia) and gel filtration size exclusion chromatography on a Superose 12 column (Pharmacia) in the wild-type P-450cam and the C58A, C85A, C136A and C148A mutants. On the other hand, no dimer was detected, even at high concentrations (0.1mM range), for the C334A mutant (see data in Figure 2). We concluded that wild-type P-450cam underwent dimerisation by intermolecular S-S

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disulphide bond formation between the surface cysteine 334 on two protein molecules.

The C334A mutation has the obvious benefit of removing unwanted protein dimerisation, thus ensuring the presence of a single species in solution at all times. In addition, we noted a completely unexpected benefit of this mutation. Like all proteins, wild-type P-450cam shows aggregation upon standing. The reasons why proteins aggregate are not clear, but the P-450cam aggregates are insoluble and catalytically inactive. The wild-type and C58A, C85A, C136A and C148A mutants all showed dimerisation as well as aggregation upon storage at 4°C, and even in 50% glycerol solutions at -20°C. Aggregation will also occur during turnover, especially at the higher P-450cam concentrations required in any economically viable industrial application in, for example, synthesis of organic molecules. The C334A mutant did not show any evidence of aggregation even at mM concentrations at room temperature over a period of three days. Thus, the C334A mutation has beneficial effects in protein handling, storage, and increased catalyst lifetime.

We believe the mutation at position 96 to be the key which enables the mutant enzymes to catalyse the oxidation of a relatively wide range of organic substrates. Other amino acids adjacent to the active site of the enzyme may also be mutated in order to change the shape and specificity of the active site. These other amino acids include those at positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396. It is envisaged that

the amino acid at one or more of these positions may be replaced
by: a small hydrophobic amino acid so as to enlarge the active
site; or a large hydrophobic amino acid so as to reduce the size
of the active site; or by an amino acid having an aromatic ring
to interact with a corresponding aromatic ring of a substrate.

Regarding the oxidation reactions, the conditions are described
in the literature references attached.   The enzyme system
typically includes putidaredoxin and putidaredoxin reductase
together with NADH as co-factors in addition to the mutant
enzyme. The example of cyclohexylbenzene oxidation is described
in the experimental section below. Various classes of organic
compounds are envisaged and described below. We note that the
wild-type P-450cam is active towards the oxidation of a number
of molecules included in the following sections. However, in
all cases the mutant P-450cam proteins show much higher turnover
activities.

i)   The organic compound is an aromatic compound, either a
      hydrocarbon or a compound used under conditions in which it
      does not inactivate or denature the enzyme.   Since the
      mutation has been effected with a view to creating an
      aromatic-binding pocket in the active site of the enzyme,
      the mutant enzyme is capable of catalysing the oxidation of
      a wide variety of aromatic compounds. Oxidation of example
      aromatic and polyaromatic compounds is demonstrated in the
      experimental section below and is believed very surprising
      given that the wild-type enzyme has been reported to

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catalyse the oxidation of only members of the camphor family and shows low activity towards a few other molecules such as styrene[19], ethylbenzene[9,10], a tetralone derivative[20], and nicotine[21].

ii) The organic compound may be a hydrocarbon, e.g. aliphatic or alicyclic, carrying a functional group (see Scheme 1). An aromatic protecting group is attached to the functional group prior to the oxidation reaction and removed from the functional group after the oxidation reaction. A suitable aromatic group is a benzyl group. The protecting group serves two purposes: firstly it makes the substrate more hydrophobic and hence increases binding to the hydrophobic enzyme pocket; secondly it may help to hold the substrate in place at the active site. Thus, with the correct aromatic protection group, both regio- and stereo-selective hydroxylation of the substrate may be achieved. Examples of monofunctionalised hydrocarbons are cyclohexyl, cyclopentyl and alkyl derivatives (Scheme 1). The oxidation products of these compounds are valuable starting materials for organic synthesis, particularly when produced in a homochiral form. A range of aromatic protecting groups are envisaged, e.g. benzyl or naphthyl ethers and benzoyl ethers and amides (Scheme 1). Of interest are also benzoxazole groups as carboxyl protecting groups and N-benzyl oxazolidine groups as aldehyde protecting groups. Both can be easily cleaved after the enzymatic oxidation

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and have previously been described in the literature for the microbial oxidations of aldehydes and acids[22].

iii) The organic compound is a C4 to C12 aliphatic or alicyclic hydrocarbon. Oxidation of cyclohexane and linear and branched hydrocarbons is demonstrated in the experimental section below. We have found that wild-type P-450cam is also capable of oxidising these molecules, but the activities are low and in all cases the mutants show substantially higher activities.

iv) The organic compound is a halogenated aliphatic or alicyclic hydrocarbon. Oxidation of lindane (hexachlorocyclohexane) is also describe below.

Mutants were constructed in which active site substitutions were combined with the surface mutation of cysteine at position 334 to alanine and contained alanine, leucine, valine, or phenylalanine instead of tyrosine at position 96 (Y96). Lastly several active site mutations and the surface mutation were combined to constitute mutant enzymes with multiple mutations. The genes encoding cytochrome P-450cam, and its natural electron-transfer partners putidaredoxin and putidaredoxin reductase, were amplified from the total cellular DNA of P. Putida using the polymerise chain reaction (PCR). The expression vector/E. coli host combinations employed were pRH1091[23] in strain JM109 for P-450cam, pUC 118 in strain JM109 for putidaredoxin, and pGL W11 in strain DH5 for putidaredoxin

reductase. Oligonucleotide-directed site-specific mutagenesis was carried out using an M13 mp 19 subclone by the method of Zoller and Smith[24], and mutant selection was by the method of Kunkel[25].

Binding of potential substrates was investigated by spectroscopic methods. The wild-type enzyme in the absence of substrate is in the 6-co-ordinated, low-spin form with a weakly bound water occupying the sixth co-ordination site, and shows a characteristic Soret maximum at 418 nm. Binding of camphor and the substrate analogues adamantanone, adamantane and norbornane fully converted the haem to the 5-co-ordinated, high-spin form which has a characteristic Soret band at 392 nm. This haem spin-state shift is accompanied by an increase in the haem reduction potential which enables the physiological electron-transfer partner putidaredoxin to reduce P-450cam and initiate the catalytic hydroxylation cycle[26]. The haem spin state shift is thus a qualitative indication of the likelihood of molecules shown in Tables 1 and 2 being oxidised by the wild-type and mutant P-450cam enzymes.

A buffered solution (50 mM Tris.HCl, pH 7.4), typically 3ml in volume, containing 10uM putidaredoxin, 2 uM putidaredoxin reductase, 1 uM cytochrome P-450cam mono-oxygenase (wild-type or mutant), 200 mM KCl, 50 ug/ml bovine liver catalase (Sigma), and 1 mM target organic compound such as cyclohexylbenzene (added as a 0.1 M stock in ethanol) was preincubated at 30°C for 5 minutes. The enzymatic reaction was initiated by adding NADH to a total

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concentration of 2 mM. Further four aliquots of NADH (to
increase the NADH concentration by 1mM each time) were added in
intervals of 10 minutes, and 30 minutes into the incubation one
aliquot of substrate (to increase the concentration by 1mM) was
also added. The reaction was quenched after 60 minutes by
adding 0.5 ml chloroform and vortexing the mixture. The phases
were separated by centrifugation (4000 g) at 4°C. The chloroform
layer was analyzed by gas chromatography.

For many substrate compounds such as cyclohexylbenzene for which
not all the P-450cam-mediated oxidation products are
commercially available, the chloroform extracts are evaporated
to dryness under a stream of nitrogen. The residues were
extracted with hexane and the oxidation products separated by
high performance liquid chromatography, eluting with a
hexane/isopropanol gradient. The purified products were then
identified by mass spectroscopy and particularly nuclear
magnetic resonance spectroscopy.

For different substrates of different solubility in the aqueous
buffer solution, the amount of substrate added to the incubation
mixtures varies from 0.2 mM to 4 mM final concentration. The
NADH concentration can be monitored at 340 nm and, in all cases,
more substrates and NADH are added during the incubation.

Using the above experimental techniques, the inventors have
investigated a considerable number of organic compounds as

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substrates for both the wild-type P-450cam enzyme and also the mutant version Y96A. Work has included mutants designated Y96V; Y96L; Y96f: C334A; the combined mutant F87A-Y96G-F193A and the combined active site and surface mutants of Y96A-C334A; Y96V-C334A; Y96L-C334A;Y96F-C334A;F87A-Y96G-F193A-C334A. The results for C334A and C334A-Y96A are set out in Table 1 and 2, in which structurally related molecules are grouped together.

Table 1 details the NADH consumption for oxidation of small linear, branched and cyclic hydrocarbons by the mutant Y96A-C334A. Tables 2(a) to 2(h) details the product distributions for mutant and substrate combinations where these have been elucidated to date.

The cysteine residue at position 344 can be deleted by any well known and freely available standard restriction techniques and will therefore not be described in detail herein.

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Scheme 1:

Hydrocarbons					
-Z		Protecting Group			
-OH					
-NH₂					
-COOH					
-CHO					
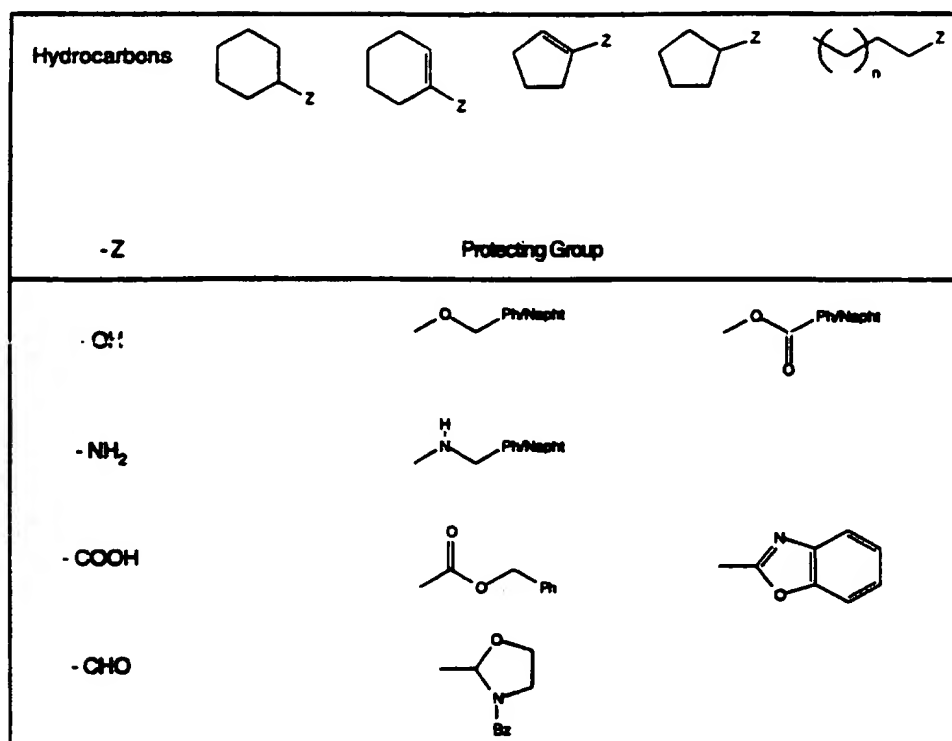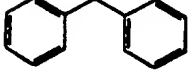
Table 1:

| | | K_{app} (μM)[a] | |
		WT	Y96A
	1	6.3	12
	2	12	28
	3	8.4	1.4
	4	330	92
	5	>1500[b]	73

[a] Values are the average of two independent measurements using the method of Sligar (S.G. Sligar, *Biochemistry*. 1976. *15*. 5399 - 5406). The value of K_{app} is strongly dependent on the concentration of K^+ in the buffer. At $[K^+]$>150 mM, K_{app} for camphor is 0.6 μM for both wildtype and Y96A. Data in this table were determined at $[K^+]$ = 70 mM in phosphate buffer. pH 7.4, in order to avoid salting out of substrates at higher ion concentrations.

[b] Saturation not reached.

Table 2(a)
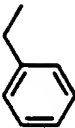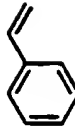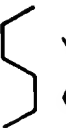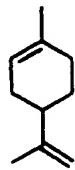
P450cam-substrate interactions

Subgroup: 1-ring

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
Benzene	-	-	-	-				
Toluene	-	-	30	30				
Ethylbenzene	-	-	40	40				
Styrene	-	-	30	30				
Cyclohexene	-	5	40	40				
1,3-Cyclohexadiene	nd	nd	nd	nd				
1,4-Cyclohexadiene	-	5	15	20				
Cyclohexane	-	-	60	60			+	
Hexane	-	-	70	60			+	
Methylcyclohexane	50	50	100	70				
(S)-(+)-Carvone	10	60	10	80				

Table 2(b)

P450cam-substrate interactions

Subgroup: 2-ring, Naphthalene

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
Naphthalene	-	-	15	20				
1-Ethylnaphthalene	-	-	5	20				
2-Ethylnaphthalene	-	-	10	20				
2-Naphthylacetate	-	5	-	5				
1-Naphthylacetate	-	5	-	5				
1-Naphthylpropionate	-	20	0	20				
1-Naphthylbutyrate	-	5	-	5				
Naphthylphenylketone	-	5	-	5				
1,2-Dihydronaphthalene	5	20	30	90				
1,2,3,4-Tetrahydronaphthalene	5	10	40	40				

Table 2(c)
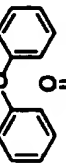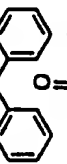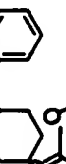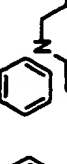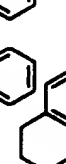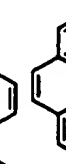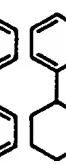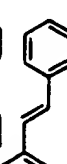
P450cam-substrate interactions Subgroup: 2-ring, DPM	Wild type ΔSpin high/low	Wild type Vs DTT	Mutant Y96A ΔSpin high/low	Mutant Y96A Vs DTT	Wild type NADH turnover?	Wild type GC?	Mutant Y96A NADH turnover?	Mutant Y96A GC?
Diphenylmethane	-	5	45	nd			+	+
Diphenylether	10	5	20	50				
Benzophenone	-	20	-	20				
Cyclohexylphenylketone-	-	30	60	nd				
Phenylbenzoate	-	5	-	.				
N-Phenylbenzylamine	-	5	45	nd				
Bibenzyl	-	.	55	55				
cis-Stilbene	-	20	40	50				
Biphenyl	-	20	80	90				
Cyclohexylbenzene	20	20	80	nd				
trans-Stilbene	-	.	.	.				
Benzylether	-	5	55	nd				

Table 2(d)
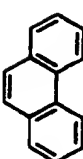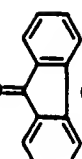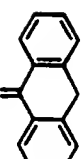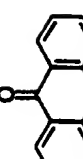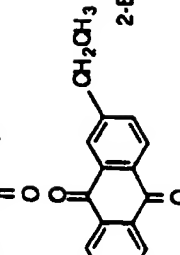
P450cam-substrate interactions

Subgroup: 3-ring

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
Anthracene	-	-						
Phenanthrene	-	-	20	20				
Fluorene	-	-	-	50				
2-Fluorencarboxaldehyde	-	-	-	50				
9-Fluorenone	-	20	-	5				
Anthrone	-	5	-	5				
Anthraquinone							+	
2-Ethylanthraquinone								

Table 2(e)
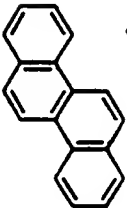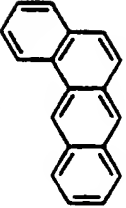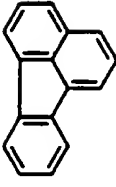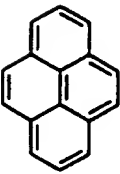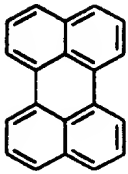
P450cam-substrate interactions Subgroup: 4,5-ring	Wild type ΔSpin high/low	Wild type Vs DTT	Mutant Y96A ΔSpin high/low	Mutant Y96A Vs DTT	Wild type NADH turnover?	Wild type GC?	Mutant Y96A NADH turnover?	Mutant Y96A GC?
Chrysene	·	·	·	·				
1,2-Benzanthracene	·	·	·	·				
Fluoranthene	·	5	20	10				
Pyrene°	·	·	·	·				
Perylene°	·	·	·	·				

Table 2(f)
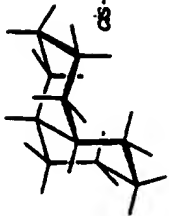
P450cam-substrate interactions

Subgroup: Cyclic Alkanes

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
cis-Decahydronaphthalene	nd	nd	nd	nd				
trans-Decahydro naphthalene	20	10	90	70			+	
Cyclohexane	·	·	60	60				
Methylcyclohexane	50	50	100	70				
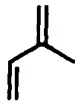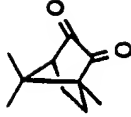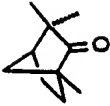
Table 2(g)

P450cam-substrate interactions

Subgroup: n-Alkanes

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
n-Pentane	-	5	55	40			+	
n-Hexane	-	-	60	40			+	
n-Heptane	5	5	60	40			+	
n-Octane	-	5	80	45			+	
n-Nonane	-	-	70	45			+	
n-Decane	nd	nd	nd	nd				
n-Undecane	nd	nd	20	20				
n-Dodecane	nd	nd	5	5				
$CH_3(CH_2)_{14}CH_3$ n-Hexadecane	-	-	-	·				
$CH_3(CH_2)_{15}CH_3$ n-Heptadecane	-	-	-	-				
$CH_3(CH_2)_{11}OSO_3Na$ SDS	-	20	-	60				
$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$ Oleic acid*	-	10?	-	20?				
$[(CH_3)_2CH(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)CH_2CH_2-]_2$ Squalane	-	-	-	20				
Isoprene	-	-	10	10				

Table 2(h)

P450cam-substrate interactions

Subgroup: Camphor-like

	Wild type		Mutant Y96A		Wild type		Mutant Y96A	
	ΔSpin high/low	Vs DTT	ΔSpin high/low	Vs DTT	NADH turnover?	GC?	NADH turnover?	GC?
(1R)-(-)-Camphorquinone	80	80	80	80				
(1R)-(-)-Fenchone	40	70	50	80				
Dicyclopentadiene	50	80	90	80				

.Table 3.

Turnover f Small Alkanes by P450cam Mutants

All mutants listed below also c ntain the C334A mutation.

Turnover rate measured as NADH consumption rate (nmole NADH/nmole P450cam/s).

Alkane substrate: Main chain length	Name	Wild type	Y96A
C4	*n*-butane	-	-
C4	2-methyl butane	background	4.6
C4	2,3-dimethyl butane	background	16.8
C4	2,2-dimethyl butane	background	14.0
C5	*n*-pentane	background	5.8
C5	2-methyl pentane	3.8	11.7
C5	3-methyl pentane	1.3	14.2
C5	2,4-dimethyl pentane	0.2	12.6
C5	2,2-dimethyl pentane	5.2	12.8
C5	2,2,4-trimethyl pentane	0.9	5.3
C5	3-ethyl pentane	background	16.2
C6	*n*-hexane	background	6.0
C6	2-methyl hexane	background	10.6
C7	*n*-heptane	2.7	4.4
C7	2-methyl heptane	background	2.1
C7	4-methyl heptane	1.4	10.2
C8	*n*-octane	background	5.8
C7	cycloheptane	4.4	42.5

Product structures and distributions following oxidation of substrates with P450cam active site mutants.

"background" - typical background NADH oxidation rate is 0.07 nmole NADH (nmole P450cam)$^{-1}$ sec^{-1}

Table 4(a)

Product structure and distributions following oxidation of substrates with P450cam active site mutants. All mutants shown below also contain the C334A mutation.

Cyclohexylbenzene Products		WT	Products (%) for mutants:			
			Y96A	Y96F	Y96L	Y96V
D or L / L or D	3-ol	43	20	54	38	28
	3-ol	20	20	27	23	39
	Trans-4-ol	25	15	6	23	10
	Cis-4-ol	12	45	13	16	23
Total products(area/10³)		0.8	7.4	1.1	10.4	12.5

Cyclohexylbenzene

P450cam

chemically most reactive position

D or L

+
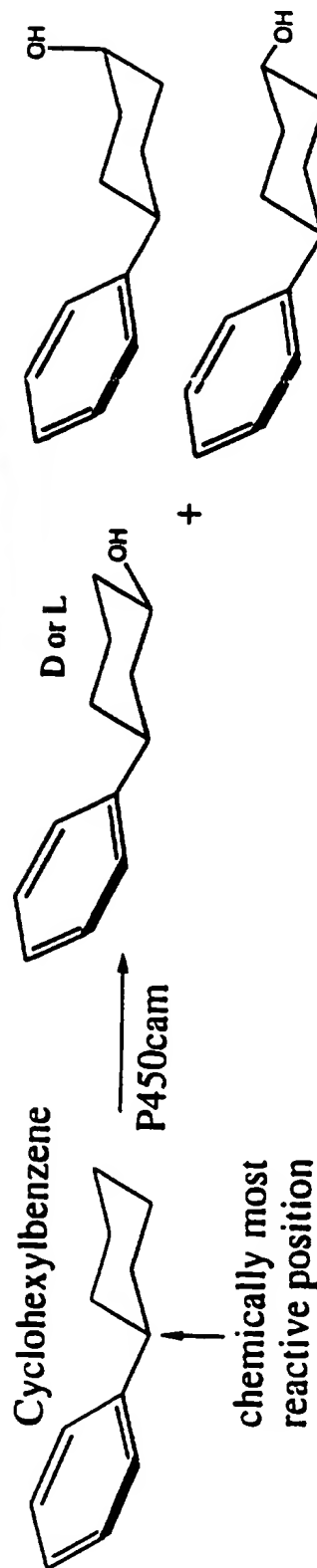
Table 4(b)

Phenylcyclohexene	Products (%) for mutants:	
Products	WT	Y96A
3-one (A)	24	25
3-ol (B)	76	75
Total products(area/10⁶)	42	36

chemically reactive positions

chemically reactive positions

P450cam

or?

Table 4(c)
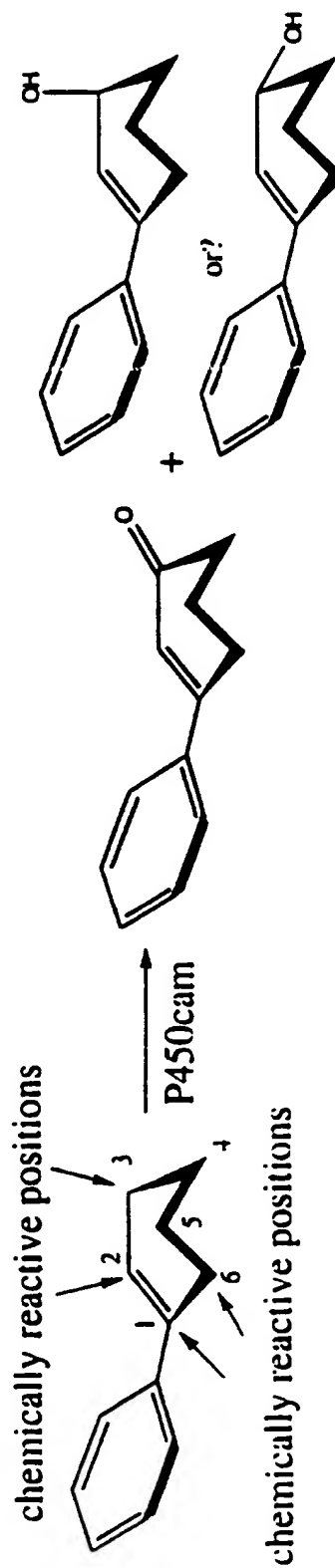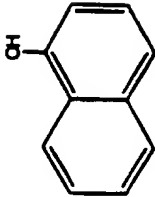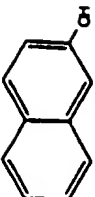
Products (%) for mutants:

Naphthalene Products	WT	Y96A	Y96F	Y96L	Y96V	F87A-F96G-F193A
1-ol	100	100	100	100	100	100
2-ol	0	0	0	0	0	0
Total products (area/10^5)	(0.016)	1.1	2.4	0.7	1.4	0.1
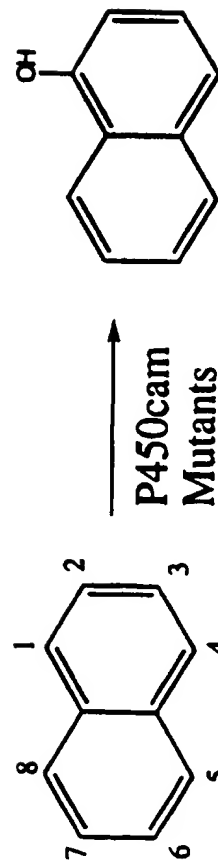
Table 4(d)

Phenanthrene Products	Products (%) for mutants:					
	WT	Y96A	Y96F	Y96L	Y96V	F87A-F96G-F193A
A	38	49	41	35.5	41	27
B	15	23	31	41	38	41
C	12	13	5	9	11	3
D	35	15	23	14.5	10	29
Total products (area/10^6)	0.075	7.0	4.5	2.8	1.6	0.065
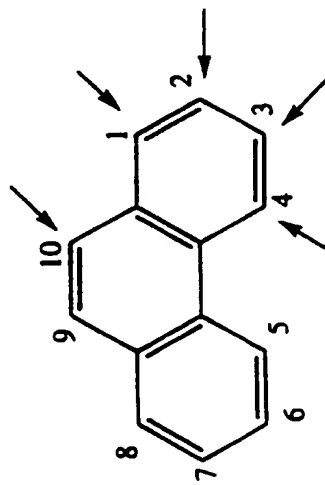
Phenanthrene $\xrightarrow{\text{P450cam mutants}}$ 4 hydroxylated products

Table 4(e)

Fluoranthene Products	Products (%) for mutants:						
	WT	Y96A	Y96F	Y96L	Y96V	F87A-F96G-F193A	
A	0	84	-	-	-	0	
B	0	16	-	-	-	100	
Total products (area/10^6)	0	2.7	-	-	-	0.2	

Fluoranthene $\xrightarrow{\text{P450cam mutants}}$ 2 hydroxylated products

Fluoranthene
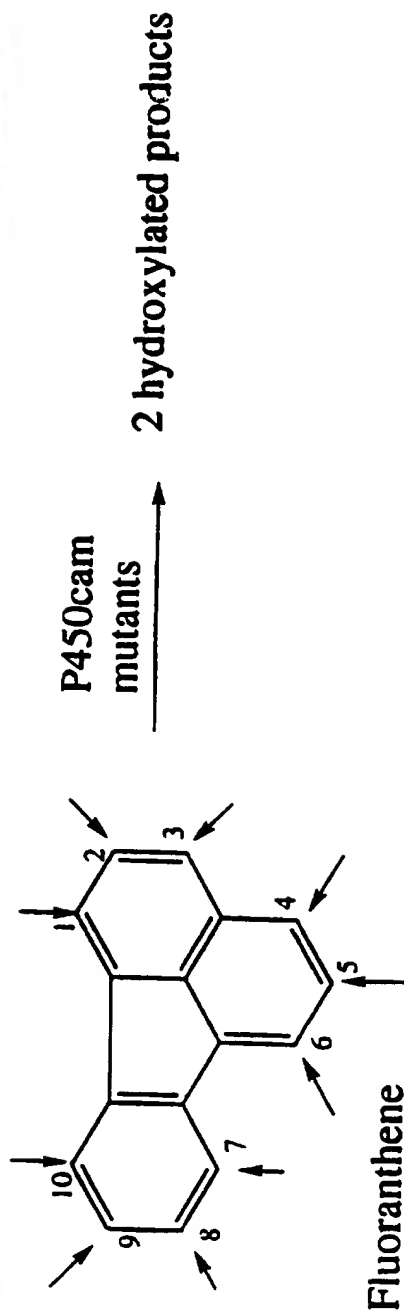
Table 4(f)

Pyrene Products	Products (%) for mutants:						
	WT	Y96A	Y96F	Y96L	Y96V	F87A-F96V	F96G-F193A
A	0	40	43	23	30	33	
B	0	43.6	29	64.5	55	40	
C	0	5	12.5	7.9	12	20	
D	0	11.4	15.5	4.6	3	7	
Total products (area/10^6)	0	1.2	1.5	1.5	1.6	0.02	

Pyrene $\xrightarrow{\text{P450cam mutants}}$ 4 hydroxylated products

Pyrene
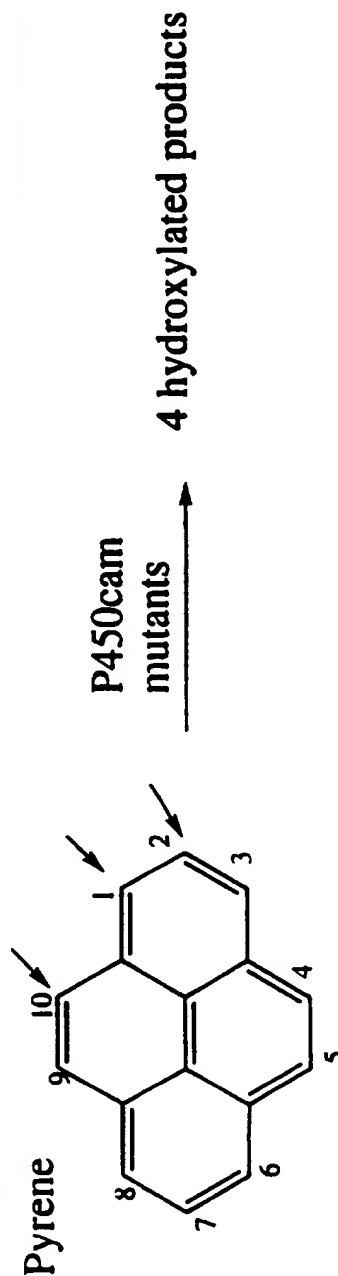
Table 4(g)

Lindane Products (hexachlorocyclohexane)

	Products (%) for mutants	
	WT	Y96A
A	100	100
Turnover rate nmole NADH (nmoleP450)$^{-1}$s^{-1}	7.5	43.5

Hexachlorocyclohexane

P450cam
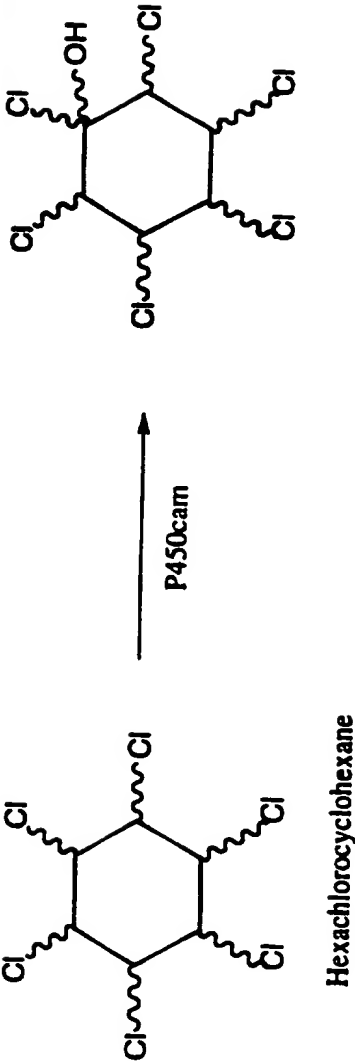
Table 4(h)

Hexane Products	Products (%) for mutants:	
	Y96F	Y96A
2-hexanone	10	15
3-hexanone	16	28
2-hexanol	24	26
3-hexanol	50	32
Relative activity (WT = 1)	18.2	25.5

2-Methyl hexane Products	Products (%) for mutants:	
	Y96F	Y96A
2-methyl-2-hexanol	72	74
5-methyl-2-hexanone	16	14
2-methyl-3-hexanol	7	4
5-methyl-2-hexanol	5	8
Relative activity (WT = 1)	2.3	2.6

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REFERENCES

1. "Cytochroe P-450: Structure, Mechanism, and Biochemistry", ed. P R Ortiz de Montellano, Plenum Press, New York, 1986.

2. T L Poulos, B C Finzel and A J Howard, J. Mol. Biol., t987, 195, 687-700.

3. C A Hasemann, K G Ravichandran, J A Peterson, and J Deisenhofer, J. Mol. Biol., 1994, 2-)6, 1169-1185.

4. K G Ravichandran, S S Boddupalli, C A Hasemann, J A Peterson, and J Deisenhofer, Science, 1993, 261, 731-736.

5. B P Unger, I C Gunsalus, and S G Sligar, J. Biol. Chem., 1986, 261, 1158-1163; J S Miles, A W Munro, B N Rospendowski, W E Smith, J McKnight, and A J Thomson, Biochem. J., 1992, 288, 503-509; T H Richardson, M J Hsu, T Kronbach, H J Bames, G Chan, M R Waterman, B Kemper, and E F Johnson, Arch. Biochem. Biophys., 1993, 300, 510-516; S S Boddupalli, T Oster, R W Estabrook, and J A Peterson, J. Biol. Chem., 1992, 267, 10375-10380; H Li K Darish and T L Poulos. J. Biol. Chem., 1991, 266, 11909-11914.

6. 1 C Gunsalus and G C Wagner, Methods Enzymol., 1978, 52, 166-188.

7. M Haniu, L G Armes, K T Yasunobu, B A Shastry, and I C Gunsalus. Biol. Chem., 1982, 257, 12664-12671.

8. S G Sligar, D Filipovic, and P S Stayton, Methods Enzymol., 1991, 206, 31-49.

9.   P J. Loida and S G Sligar, Biochemistrv, 1993, 32, 11530-
      11538.

10.   P J Loida and S G Sligar, Protein Eng., 1993, 6, 207-212.

11.   W M. Atkins and S G Sligar, J. Am. Chem. Soc., 1989,
      111, 2715-2717.

12.   S F Tuck, S Graham-Lorence, J A Peterson, and P R Ortiz
          de Montellano, J.Biol. Chem., 1993, 268, 269-275.

13.   C Di Prime, G Hui Bin Hoa, P. Douzou, and S Sligar, J.
      Biol. Chem., 1990, 265, 5361-5363.

14.   W M Atkins and S G Sligar, J. Biol. Chem., 1988, 263,
      18842-18849.

15.   W M Atkins and S G Sligar, Biochemistry, 1990, 29, 1271-
      1275.

16.   J D Lipscomb, J E Harrison, K M Dus, and I C Gunsalus,
      Biochem. Biophys. Res. Conunun., 1978, 83, 771-778.

17.   P J Goodford, J. Med. Chem., 1985, 28, 849-857.

18.   Quanta 4.0, Molecular Simulations Inc., 16 New England
      Executive Park, Burlington, MA 01803-5297.

34

19.   J A Fruetet, J R Collins, D L Camper, G H Loew, and P R
      Ortiz de Montallano, J. Am. Chem. Soc., 1992, 114,
      6987-6993.

20.   Y Watanabe and Y Ishimura, J. Am. Chem. Sec., 1989,
      111, 410-41 1.

21.   J P Jones, W F Trager, and T J Carlson, J. Am. Chem.
      Soc., 1993, 115, 381-387.

22.   "Biotransformation in Preparative Organic Chemistry" H G
      Davis, R H Green, D R Kelly, and S M Roberts, Academic
      Press, London, 1989, Page 169 ff.

23.   J E Baldwin J M Blackburn, R J Heath, and J D Sutherland,
      Bioorg. Med. Chem. Letts. 1992, 2, 663-668.

24.   M J Zoller and M Smith, Nucleic Acids Res., 1982, 10,
      6487-6500.

25.   T A Kunkel, Proc. Natl. Acad. Sci., USA 1985, 82, 488-
      492.

26.   S G Sligar and I C Gunsalus, Proc. Natl. Acad. Sci., USA,
      1976, 73, 1078-1082.

CLAIMS

1. A mutant of the mono-oxygenase cytochrome P-450cam in
    which the cysteine residue at position 334 is removed.

2. A mutant as claimed in claim 1 in which the removal is by
    the substitution of another amino acid except cysteine
    for the cysteine residue.

3. A mutant as claimed in claim 1 in which the removal is by
    deletion of the entire cysteine 334 residue from the
    enzyme.

4. A mutant as claimed in any of the preceding claims in
    which the tyrosine residue at position 96 in the mutant
    is replaced by any other amino acid except tyrosine.

5. A mutant as claimed in either of claims 1, 2 or 4 in
    which the amino acid is selected from any one of the
    following:-

    alanine, arginine, asparagine, aspartic acid, glutamic
    acid, glutamine, glycine, histidine, isoleucine,
    leucine, lysine, methionine, proline, serine, threonine,
    tryptophan, tyrosine and valine.

6.   A mutant as claimed in any of the preceding claims in
      which the amino acid residue at one or more of the
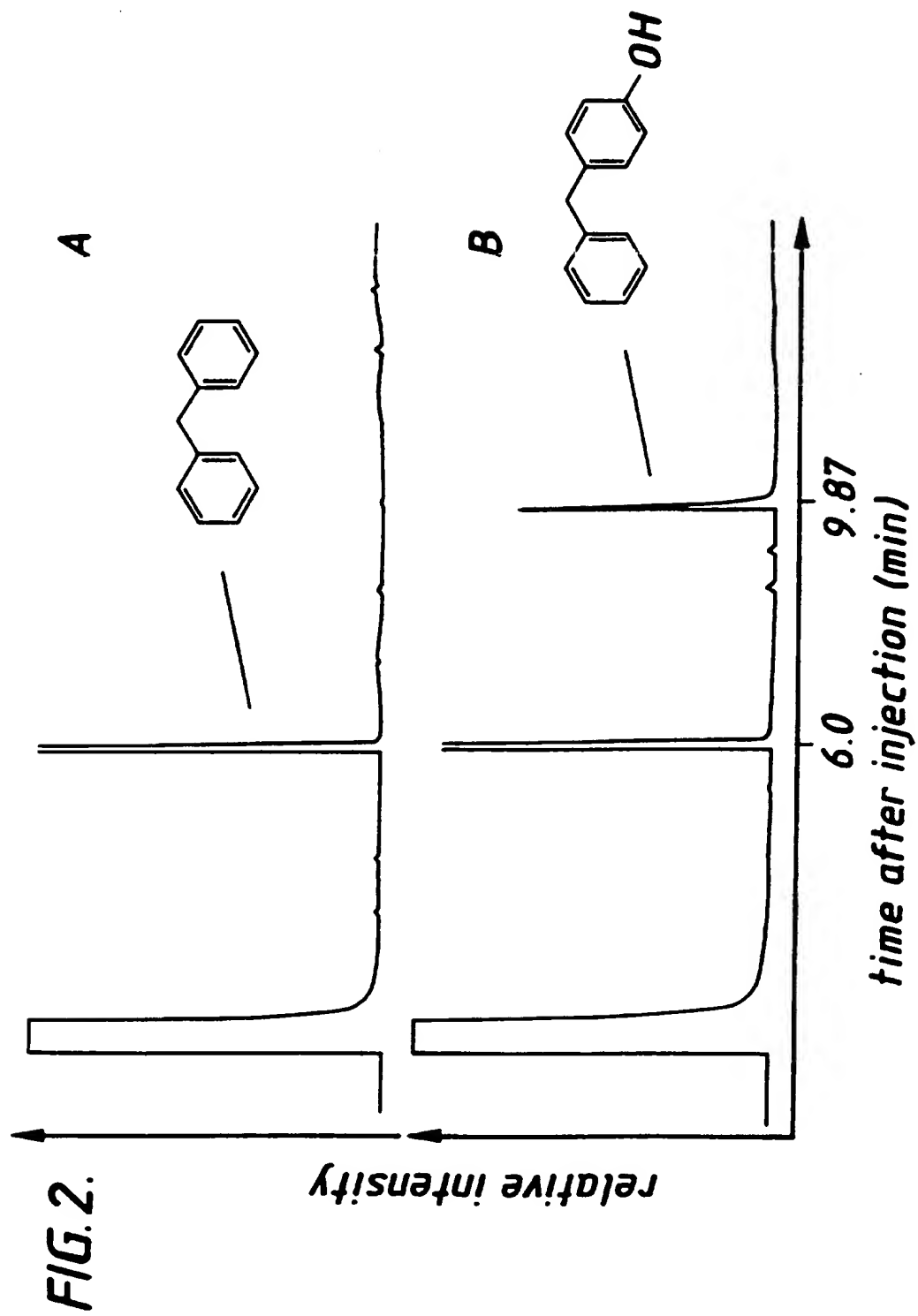   positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395
      and 396 is replaced by another amino acid residue.

7.   A mutant of the mono-oxygenase cytochrome P-450cam
      substantially as hereinbefore described with reference to
      the accompanying drawings and/or examples.

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FIG.1.
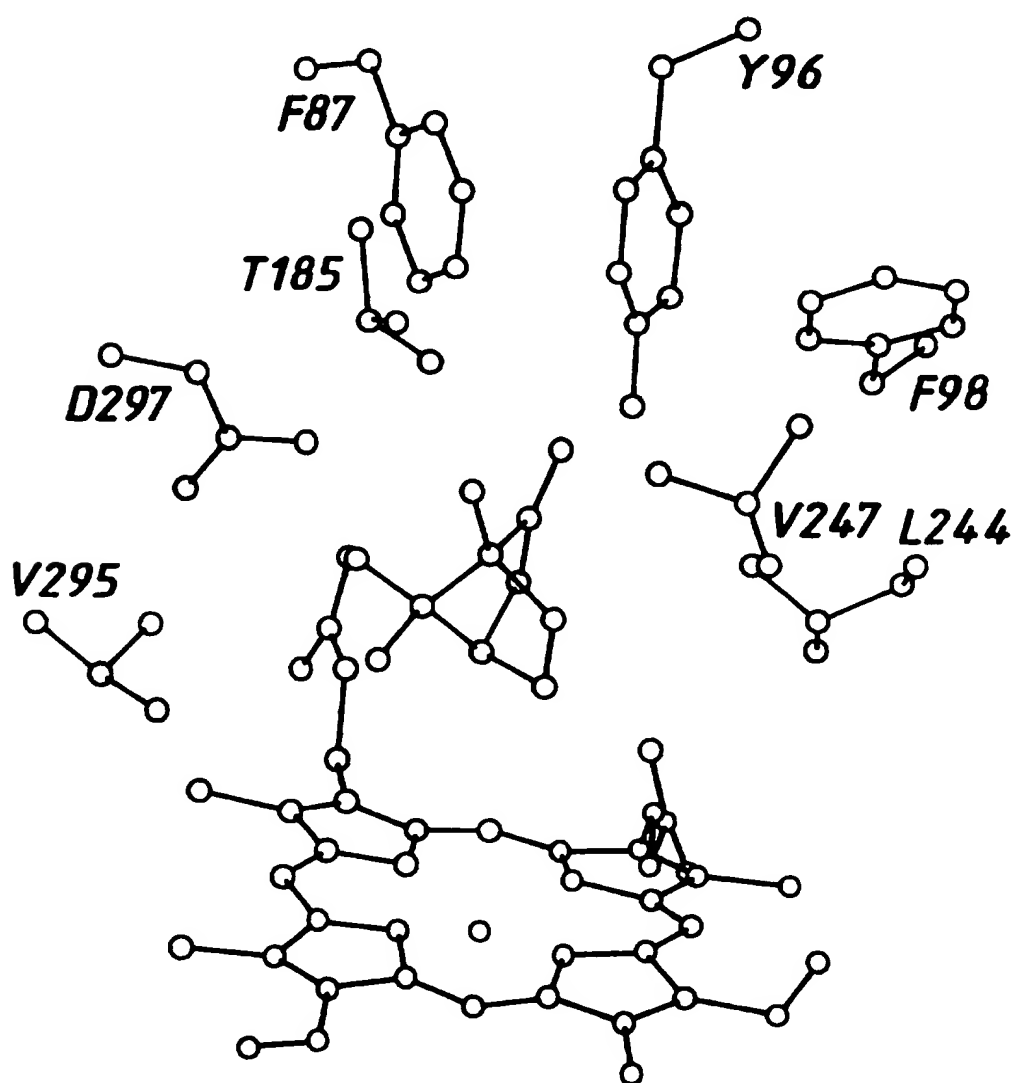
2/2

FIG.2.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/53   C12N9/02   C12P7/22   C12P7/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 35, 15 December 1988, pages 18842-18849, XP002025958 W.M. ATKINS ET AL.: "The role of active site hydrogen bonding in cytochrome P-450 cam as revealed by site-directed mutagenesis." cited in the application	7
Y	see the whole document ---	1-6
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 10, 5 April 1990, pages 5361-5363, XP002025959 C. DI PRIMO ET AL.: "Mutagenesis of a single hydrogen bond in cytochrome p450 alters cation binding and heme solvation." cited in the application	7
Y	see the whole document ---	1-6

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1997	1 4. 03. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hix, R

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY, vol. 29, no. 5, 6 February 1990, pages 1271-1275, XP002025960 W. M. ATKINS ET AL.: "Tyrosine-96 as a natural spectroscopic probe of the cytrochrome P-450cam active site" cited in the application	7
Y	see the whole document	1-6
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 1, 5 January 1993, pages 269-275, XP002025961 S. F. TUCK ET AL.: "Active sites of the cytochrome p456cam {CYP101} F87W and F87A mutants." cited in the application see the whole document	1-6
P,Y	WO 95 34679 A (US HEALTH ;GONZALEZ FRANK J (US); IDLE JEFFREY R (GB)) 21 December 1995 see the whole document	1-6
A	WO 95 16041 A (CIBA GEIGY AG ;ROYAL VETERINARY & AGRICULTURA (DK); KOCH BIRGIT MA) 15 June 1995 see the whole document	1-7
A	PSYCHIATRIC GENETICS 4 (4). 1994. 215-218. ISSN: 0955-8829, XP000565684 DAWSON E ET AL: "An association study of debrisoquine hydroxylase (CYP2D6) polymorphisms in schizophrenia." see the whole document	1-7
A	THE LANCET, vol. 339, no. 8806, 6 June 1992, pages 1375-1377, XP000565682 C.A.D. SMITH ET AL: "Debrisoquine hydoxylase gene polymorphism and susceptibility to Parkinson's disease." see the whole document	1-7
X,P	WO 96 14419 A (BRITISH GAS PLC ;FLITSCH SABINE LAHJA (GB); NICKERSON DARREN PAUL) 17 May 1996 see the whole document	1-7

2

International Application No

PCT/GB 96/02693

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534679 A	21-12-95	AU 2860295 A	05-01-96
WO 9516041 A	15-06-95	AU 1108295 A CA 2176949 A EP 0733112 A	27-06-95 15-06-95 25-09-96
WO 9614419 A	17-05-96	AU 3811795 A GB 2294692 A	31-05-96 08-05-96